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Award Number: W81XWH-07-1-0499

TITLE: Engineering Anti-EGFR Antibodies for Treatment of Breast Cancers with Poor Prognosis

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REPORT DATE: September 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September 2008	Annual	1 Sep 2007 – 31 Aug 2008

4. TITLE AND SUBTITLE

Engineering Anti-EGFR Antibodies for Treatment of Breast Cancers with Poor Prognosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0499

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, San Francisco
San Francisco, CA 94143

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
To enhance in vivo efficacy of anti-EGFR full length IgG for treatment of basal breast cancers, the most aggressive subtypes, we have successfully employed a molecular evolution approach to improve Fc binding affinity to Fc receptors that activate ADCC. In detail, both human and murine Fc domains have been functionally displayed on the surface of yeast. The Fc activating receptors, human FcγRIIIa and murine FcγRIV, were expressed in a transient 293E system and used for evaluating and sorting the affinity improved Fc domains. Libraries of Fc domains with random mutations were constructed and sorted. Several Fc domain variants were identified to bind human FcγRIIIa with enhanced affinity compared to wild-type Fc. We also found that the binding increase of Fc domain to human FcγRIIIa correlated with that to murine FcγRIV, indicating that the Fc domain with improved affinity to human FcγRIIIa should show enhanced in vivo efficacy in murine breast tumor model.

15. SUBJECT TERMS
Fc domain, Fc receptor, affinity, yeast display

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	12	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

The basal phenotype of breast cancers currently are associated with poor prognosis, partially due to the fact that there are no targeted therapies that are directed towards this type of cancer. While epidermal growth factor receptor (EGFR) is frequently overexpressed on basal type breast cancers, the response rate is not dramatic to anti-EGFR antibody therapy. One mechanism by which antibodies in general cause cancer cell death is by activating the immune system via the Fc portion of the antibody. Such activation is called antibody dependent cellular cytotoxicity (ADCC) and has been shown to be important in the anti-cancer activity of some antibodies. To determine the importance of ADCC in the response of basal breast cancers to antibody therapy, and to develop a better basal breast cancer therapeutic, we sought to develop anti-basal breast cancer antibodies with enhanced ADCC.

Specifically, to target basal subtype breast cancers with poor prognosis, we proposed to engineer EGFR full length IgG antibodies to have enhanced ADCC. To achieve this research goal, we designed to improve the affinity of the Fc portion of the IgG for activating Fc receptors using our established yeast display platform. The designed full length IgG would have a previously optimized EGFR antigen-binding fragment (Fab) and an improved constant fragment (Fc) that would be generated in this project. We anticipate that the platform generated for enhancing ADCC will work for the evolution of antibody affinity to other Fc receptors, which play important roles in modulating antibody function *in vivo*.

BODY:

Engineering anti-EGFR antibodies for treatment of breast cancers with poor prognosis
Studies and Results

Task 1. To display murine IgG1 Fc region on yeast surface (Months 1-5):

a. Synthesize murine IgG1 Fc region with compatible restriction sites for cloning into pYD2 vector.
b. Expression of murine IgG1 Fc on yeast surface using standard yeast transformation and culture.
c. Assess the binding of murine Fc on yeast surface to protein A, protein G and anti-SV5 tag for expression.
d. Expression of sFcγRIII, sFcγRIIB and sFcRn in 293E system. Alternatively, we will utilize stable expression if unsuccessful using transient expression.
e. Determine the binding affinity of murine Fc on yeast surface to sFcγRIII, sFcγRIIB and sFcRn using flow cytometry.

A-C. To validate the functional display of IgG heavy chain constant region on the yeast surface, both human and murine IgG1 CH1-hinge-CH2-CH3 and hinge-CH2-CH3 were cloned into the pYD2 vector(*1*). The yeast EBY100 transformed with such IgG1 constant region containing pYD2 constructs were induced for the surface display following the standard procedures(*2*). Both IgG1 CH1-hinge-CH2-CH3 and hinge-CH2-CH3 were displayed on the surface of yeast as determined by staining of SV5 tag at the C-terminus of the protein (Figure 1)(*3*).

To assess the proper folding of the IgG1 constant regions, protein A-FITC was used to stain the yeast and showed binding of the same percentage of yeast as that stained with SV5 antibody, which indicated that the displayed IgG1 constant region folded properly (Figure 1).

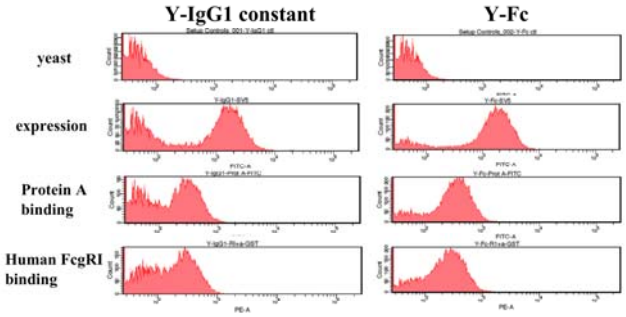


Fig. 1 Display of human IgG1 constant region and Fc region on yeast surface

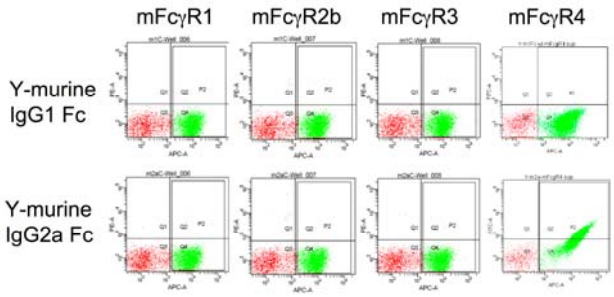


Fig. 2 Display of murine Fc region on yeast surface

Murine Fc domains from IgG1 and IgG2a were displayed on the yeast surface as assessed by staining of the SV5 tag at the domain C-terminus. The displayed murine Fc domains did not bind murine Fc gamma receptor 1, 2b and 3, only Fc domain of IgG2a bound Fc gamma receptor 4, the activating receptor in mouse(*4*). The stronger binding of IgG2a Fc domain to mFcγR4 agrees with the fact that IgG2a simulates stronger immune function than IgG1 (Figure 2).

D. To express Fc receptors efficiently, a mammlian 293E system was used for sustaining production of human Fc receptors(5). In this system, sFcγRI, sFcγRIIA, sFcγRIIB, sFcγRIIIA/V158 and sFcRn were expressed. In order to scale up, 293E cells were adapted to suspension culture, culture conditions were adjusted to optimize this transient expression. Currently, the yield of sFcγRIIIA/V158 and sFcγRIIA reached around 5mg/L culture, the yield of sFcγRIIB is still around 1mg/L culture. Among all the receptors, sFcRn is difficult

to get good yield regardless of the culture condition. For sFcRn, we will subclone into a stable transfection system for better expression yields.

Murine Fcγ receptors are commercially available except murine FcγR4, which we expressed in 293E system. However, the expression yield was so low that we could not purify for affinity determination. Murine FcRn that is not available will be expressed in stable transfection system that we use for full length IgG production.

E. Using fluorescent activated cell sorting (FACS), the binding of human Fc receptors to human IgG constant region displayed on yeast surface were detected except for the FcRn. The affinity measured by flow cytometry on yeast displayed wild type hIgG1 Fc was consistent for hFcγR1, hFcγR2a and hFcγR3a with other measurement except that the affinity for hFcγR2b was too low to be determined (Figure 3).

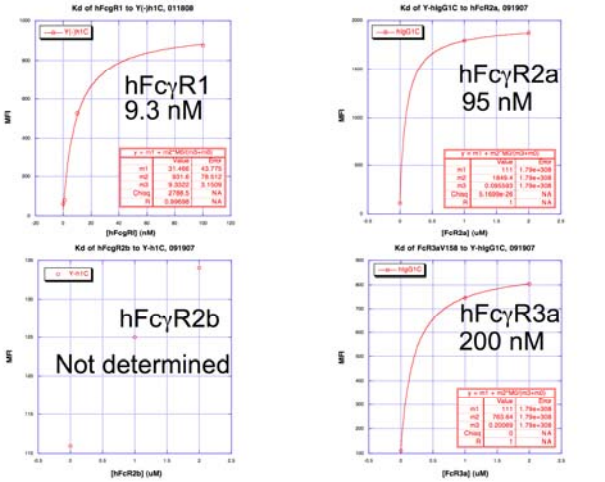


Fig. 3 Measure hFcgR affinity to Fc region displayed on yeast surface

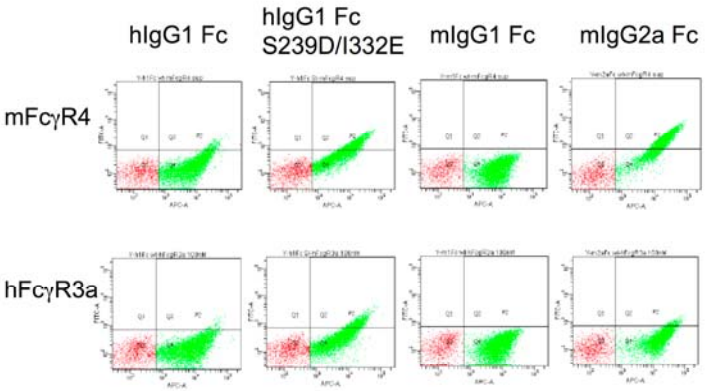


Figure 4. Binding of human and murine IgG Fc domains to both human and murine activating Fc receptors

The hIgG1 Fc variant S239D/I332E with higher affinity to sFcγRIIIA/V158 was also displayed on yeast surface and showed stronger binding to sFcγRIIIA/V158 than the wild type hIgG1(*6*). We also found that the increased binding affinity of human IgG Fc variant for sFcγRIIIA/V158 also correlated with the higher binding to murine sFcγR4 (Figure 4) although the affinity to sFcγR4 could not be determined due to the low yield of the receptor. This finding indicated that the increased binding affinity to human FcγR3a/V158 and murine FcγR4 can be achieved at the same time, and also led us to hypothesize that by improving the affinity of human IgG Fc to either human FcγR3a/V158 or murine FcγR4, the Fc variant with enhanced immune effector function in both human and murine systems can be identified. As such, we will mutate human IgG1 Fc domain to improve the affinity to both human FcγR3a/V158 and murine FcγR4 in Task 2.

Task 2. To construct human Fc variant library on yeast surface using whole gene random mutagenesis method (Months 6-9):

a. Error-prone PCR at moderate to high mutagenesis rate to introduce mutations randomly into whole murine Fc cDNA.
b. Transfer the mutated murine Fc cDNA into pYD2 by Gap repair.
c. Transform and express the mutated murine Fc region in yeast to make a library comprising 10^7-10^8 individuals.
d. Evaluate the mutation rate by DNA sequencing.
e. Evaluate the expression and binding of Fc mutant library by staining with anti-SV5 tag and protein A respectively.
f. Evaluate the binding of Fc mutant library to sFcγRIII, sFcγRIIB and sFcRn using flow cytometry.

A-D. To randomly diversify the human IgG1 Fc region at a moderate-to-high mutation rate (*7*), the Fc gene was amplified for 20 cycles under error-prone conditions with Taq polymerase and $MnCl_2$, followed by further amplification using a proof-reading polymerase for 35 cycles. The mutated Fc gene repertoire was cloned into the pYD2 vector using homologus recombination in *Saccharomyces cerevisiae* to generate a library of 2×10^7 transformants. DNA sequencing of nine Fc genes showed that each Fc gene had on average 10.7 amino acid substitutions. The location of the mutations was random, as the mutations were distributed evenly between the hinge, CH2 and CH3 regions (Figure 5).

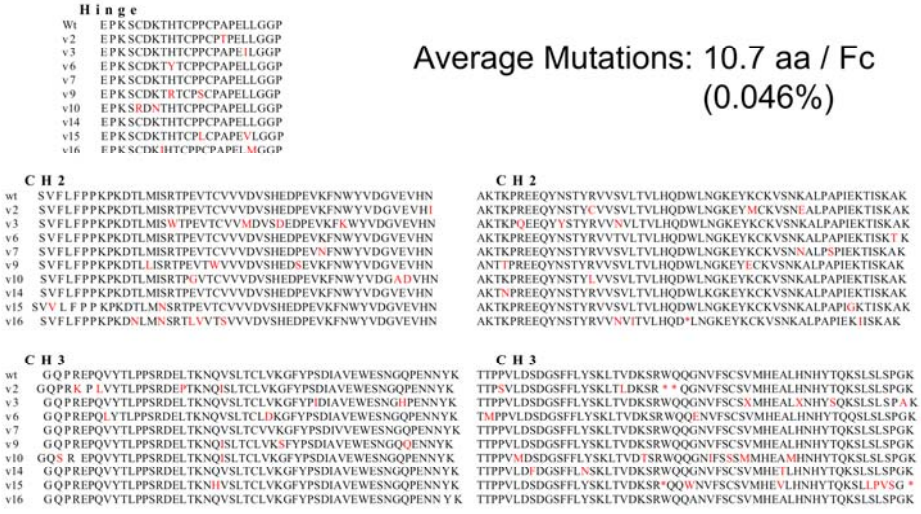


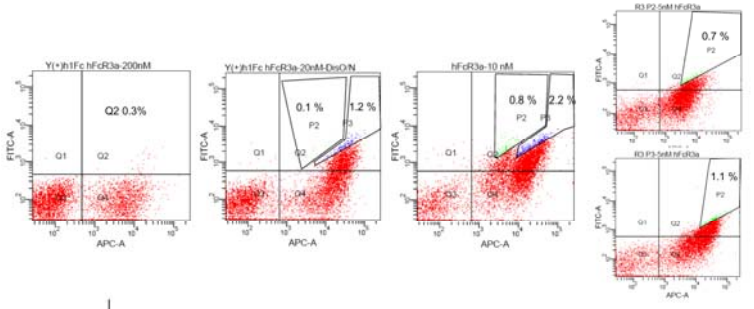
Fig 5. Error-prone Mutation of Human Fc Domain

E-F. The expression level of h1Fc variant library represented by populations in Q4 area was 19%, which was less than the parental clone that expressed 68.5%. The binding analysis of h1Fc variant library showed that 0.3% of the total yeasts bound sFcγRIIIA at 200 nM, no binding to sFcγRIIA and sFcγRIIB.

Task 3. To select Fc mutants with increased binding to sFcγRIII while decreased binding to sFcγRIIB by fluorescent activated cell sort (FACS) (Months 10-13):

a. The displayed Fc mutant library was subjected to 4 rounds of selection using sFcγRIIB for negative sorting and sFcγRIII for positive sorting.
b. Evaluate the output from 4th round of sorting for binding to sFcγRIII and sFcγRIIB.
c. Screen individual clones for increased binding to sFcγRIII and decreased binding to sFcγRIIB by flow cytometry.
d. Determine the binding affinities of selected clones to sFcγRIII and sFcγRIIB by flow cytometric analysis on yeast surface.
e. Identify selected Fc mutants by DNA sequencing.

A. Higher-affinity Fc region mutants were selected from the error-prone yeast displayed library using flow cytometry(*8*). Based on binding evaluation of the mutant library in Task 2, 200 nM sFcγRIIIA/V158 was used for the first round of sorting, with all yeast showing binding to sFcγRIIIA/V158 above background selected for recovery (0.3% of the total population) (Figure 6). To select for higher-affinity Fc, the concentration of sFcγRIIIA/V158 was decreased to 20 nM, 10 nM and 5 nM for the successive rounds of sorting(*9*). To select Fc mutants that have slow



	R1	R2	R3	R4
Input	1.04×10^8	4.25×10^6	3.4×10^6	P2 4.7×10^7 P2 3.8×10^7
Output	9.68×10^4	P2 5.51×10^3 P3 2.9×10^4	P2 1.1×10^4 P3 2.9×10^4	P2 9.7×10^4 P3 1.8×10^5

Fig 6. Sort the human Fc mutant library for improved binding to human Fc gamma receptor 3A

off rate, the mutant library recovered from the first round of sorting was subjected to short incubation with sFcγRIIIA/V158 followed by dissociation overnight. After two rounds of sorting, two populations with different expression levels were gated separately as P2 and P3, followed by another round of sorting independently (Figure 6).

B-E. The outputs from each of these sorting were grown, induced, stained with sFcγRIIIA/V158, and the MFI for sFcγRIIIA/V158 binding compared to the MFI for sFcγRIIIA/V158 binding of wild-type human Fc region. Monoclonal Fc from the P3 population showed significantly stronger sFcγRIIIA/V158 binding than wile-type Fc, suggesting that each of these scFv had higher affinity for sFcγRIIIA/V158 than the parental Fc (Figure 7). Since the binding to sFcγRIIB was too low to be measured, the enhanced binding to sFcγRIIIA/V158 will be used primarily for selecting Fc mutants.

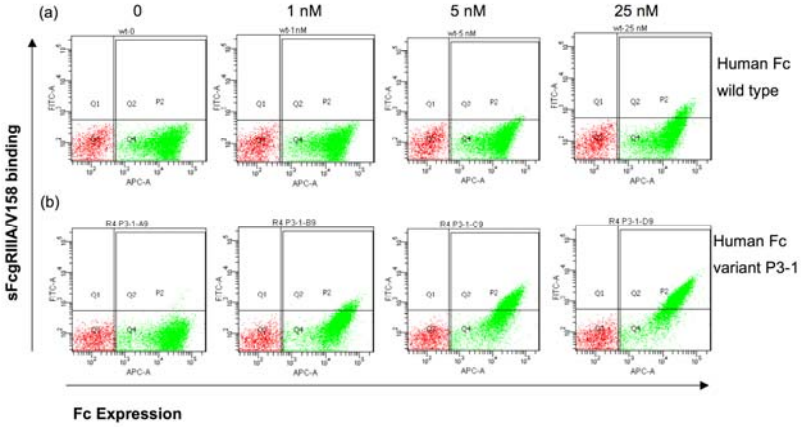


Figure 7. Binding of yeast-displayed human Fc region to human Fc receptor sFcgRIIIA/V158 by flow cytometry. (a) Wild type human IgG1 Fc region; (b) mutant P3-1 selected from round 4 sorting; bound to sFcgRIIIA/V158 at different concentration.

DNA sequencing of the monoclonal Fc mutants revealed that the location of the substitutions was not restricted to the hinge and CH2, which are involved in the binding to Fc gamma receptors. In order to avoid any change of binding to FcRn, we selected Fc mutants that have no or less mutations in CH3 for further characterizations.

For human Fc mutants P3-1, P3-3 and P3-4, the equilibrium dissociation constant (K_D) for sFcγRIIIA/V158 was determined using flow cytometry, and the best mutant showed seven fold improvement compared to wild-type Fc region displayed on yeast surface (Table 1).

Table 1. K_D of yeast-displayed human Fc regions for sFcγRIIIA/V158.

[hFcgR3a] (nM)	wt	R4 P3 -1	R4 P 3-3	R4 P3 -4
0	92	76	68	67
1	98	216	180	127
5	129	641	494	379
25	226	1165	867	865
Kd (nM)	**46**	**7.71**	**7.15**	**15.9**

KEY RESEARCH ACCOMPLISHMENTS:

1. We expressed and purified human FcγRIIIA/V158, which is not commercially available.
2. We also expressed mouse FcγRIV, which is also not commercially available.
3. Affinity maturation of Fc using yeast display was accomplished.
4. Several Fc variants were selected for the enhanced binding to human FcγRIIIA/V158 and mouse FcγRIV, two dominant activating receptors for ADCC effect.

REPORTABLE OUTCOMES

1. Presentation:

Yu Zhou, James D Marks. (2008) Engineering anti-EGFR antibodies for treatment of breast cancers with poor prognosis. Era of Hope, Baltimore, MD, June 25-27, 2008

2. Manuscripts:

Yu Zhou, Yong Tang, Louis M Weiner, James D Marks. (2008) Molecular evolution of Fc affinity for enhanced ADCC effect (In preparation)

CONCLUSION:

1. Both human and murine Fc region were successfully displayed on yeast surface, affinity for different Fc receptors measured.
2. Using 293E transient expression system, human FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA/V158, and mouse FcγRIV were expressed.
3. A human Fc random mutant library was generated, sorted, and individual variants characterized for increased binding affinity to FcγRIIIA/V158.

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